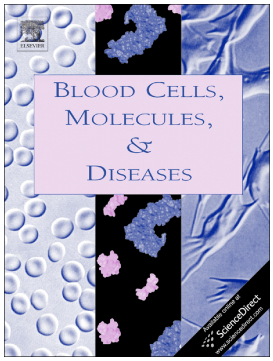
Enhancer deletion generates cellular phenotypic diversity due to bimodal gene expression



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To the editor:

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Remote *cis*-regulatory elements, such as enhancers have multiple roles with consequences of increased transcription of their target genes. Disruption of these elements can lead to alterations in gene expression, causing both Mendelian and complex diseases. Examples of such altered gene regulation, underlying the aetiology of human diseases, are the deletions in the α and β globin *cis*-regulatory elements leading to thalassemia. However the phenotypic change resulting from these genetic variations has been always evaluated in cell population-based studies. In such studies cells are believed to be, as they are genetically, homogeneous also in respect of the target-gene expression. However, cell-population expression studies lack the resolution to reveal heterogeneity at single cell level, resulting from the different way a regulatory element may act on the target gene.

Over two decades ago, several groups suggested that enhancers could act in a rheostatic mode affecting the rate of transcription of a target gene [1,2]. This model of "uniform and progressive response" of a gene to enhancer activity proposes that enhancers can increase the levels of gene expression. Another proposed model for enhancer activity, the "on or off response" model, suggests that enhancers can increase the probability a gene will become transcriptionally active and stay active. Indeed, the analysis of transcriptional activation by enhancers on a single cell basis (by using immunofluorescence or fluorescence-activated cell sorting to determine reporter gene activity) has led to the observation that enhancers increase the probability that a gene will be transcribed in any particular cell while not affecting the rate of transcription in the cells in which the gene is active [1,3-5]. Recent evidence also supports this "on or off response" model and suggests that enhancers can increase the occurrence of transcriptional bursting but not the amplitude of transcriptional burst size [6].

We previously developed a "humanised mouse" model in which the entire mouse α-globin locus is replaced by the human locus, and fully recapitulates the normal developmental pattern of α-globin expression. This model has been very valuable to address the role of the major enhancer (MCS-R2) in the expression of the -globin gene (reviewed in [7]). In the absence of MCS-R2 (ΔMCS-R2), low levels of RNA-Polymerase II are detected by chromatin immunoprecipitation (ChIP) at the α-globin gene and low levels (~1-2% of normal) of α-globin mRNA accumulate in the cells [7].

Here, we investigated the α-globin mRNA expression in ΔMCS-R2 vs normal humanised erythroblasts to determine whether this expression is derived from few cells (expressing α-globin mRNA at high levels) or from all cells (expressing α-globin mRNA at basal levels). As expected, human α-globin transcripts were detected in nearly all mouse erythroblasts containing the normal human α-globin cluster (Figure 1A). On the basis that a single normal erythroid cell may contain ~10,000 α and β-globin mRNA molecules [8] we would expect that the ΔMCS-R2 mutant (expressing ~1-2% α-globin mRNA), should contain about 100-200 copies per cell. However, we found a detectable level of fully extended α-globin transcripts in only 47% of β-globin positive mutant cells (Figure 1B) compared to 85% of normal cells.

The basal level of expression in ΔMCS-R2 mutant at either a population or a single-cell basis could reflect the transcription status at a given time. Therefore no detectable mRNA would be just a consequence of a delayed basal level of expression. We thus investigated if the accumulation of the α-globin chains in enucleated red cells would give a more homogenous pattern than mRNA transcription. Immunofluorescence staining of peripheral blood in normal vs ΔMCS-R2 mutant humanised mice was performed using an antibody specific for human α-globin chains (Suppl Figure 1). In normal humanised mice, the expression is high with a homogenous pattern in all erythroid cells (Figure 2). The use of a humanised system is thus not subjected to a variation of expression as previously observed in transgenic models (PEV) and is therefore suitable model. In the absence of MCS-R2, a heterogeneous level of α-globin chains is evident in about one third of the cells (ΔMCS-R2, Figure 2). The immunofluorescence data were confirmed by flow

cytometry analysis, which allowed us to measure more accurately a positive population of 28% and the degree of α-globin production (Suppl Figure 2).

This study describes an example where the same inherited autosomal genetic defect can generate two opposite gene-expression phenotypes at the cellular level. We have shown that in the absence of a tissue-specific enhancer element, a significant number of red cell progenitors (~50%) succeed to activate α-globin transcription (binary mode). Transcription is then retained in a smaller proportion of cells (~30%) throughout differentiation until these cells enucleate into mature red cells. This demonstrates that the active transcriptional status is maintained through cell divisions, albeit at different level thus also reflecting a rheostatic mode of gene expression.

Our results have implications also in the interpretation of studies on the epigenetic mechanisms modulating gene expression. We have previously shown that the balance of active and repressive epigenetic marks may reflect different degrees of silencing and transcription within a population of cells rather than a bivalent (H3K4me3 and H3K27me3) chromatin domain [7]. Here, we have shown evidence of the co-presence of two transcriptionally unique, and genetically identical, populations of the same cell identity. Therefore, in transcriptionally mixed cell populations, any epigenetic analysis could lead to misinterpretation of data. Recently, there have been breakthroughs in the analysis of bivalent marks using combinatorial indexed ChIP (co-iChIP) [9] [10] and single molecule modification imaging combined with sequencing [11]. However, these studies still don't address whether or not the starting material is homogenous at the expression level of a single gene. If evidence of a mixed population, with regards to the expression of a single gene (in this case, α-globin), is observed such ChIP experiments would first require a separation of these two populations. Although we made several gene-targeting attempts, the generation of a humanised α-globin reporter mouse model has proved particularly challenging due to the inherent repetitive nature of the -globin cluster.

In conclusion, this study describes a striking example where the same inherited autosomal mutation can generate two opposite transcriptional phenotypes within the

same cellular type, a phenomenon that has to be taken in account when performing epigenetic studies.

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Contribution: M.D.G. performed the research and analysed the data; A.B. drafted the manuscript; J.A.S. performed the research; J.A.S.S. performed the research, A.J.S. performed the research; W.G.W. designed and performed the research, and D.V. designed and performed the research, analysed the data, drafted and finalised the manuscript.

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Figure Legends

Figure 1. Single cell PCR analysis in humanised erythroid cells. An example of α globin expression analysis carried out in 90 mouse single erythroblasts (Ter119+). Each lane (numbered 1 to 20 and 1 to 24) corresponds to the same single cell. The expression of human α-gobin (hα) has been compared to mouse β-globin (mβ) in mouse normal (A) and in ΔMCS-R2 (B) humanised erythroblasts. The red asterisks represent no detectable expression of hα compared to mβ. The black asterisks represent no detectable expression for both mβ and hα and were not considered as informative. M = molecular weight marker (2-Log NEB). NTC = no template control. 10x cells = PCR analysis performed on 10 cells, as a control.

Figure 2. Immunofluorescence of red cells using a specific antibody against human α-globin chains. (Top) Illustrations representing the two scenarios

(rheostatic and binary modes) by which gene expression can be modulated down to 1-2% in the absence of an enhancer. (Bottom) Immunostaining with anti-human α-globin antibody (1:100) (Santa Cruz D-16 sc-31110) of mouse normal humanised red cells, mouse and human red cells at 100:1 ratio (showing species specificity of the antibody but also as an example of how an exclusive enhancer-binary mode activity should appear in ΔMCS-R2), and mouse humanised red cells without the enhancer (ΔMCS-R2). Secondary antibody was used at 1:200 dilution (Alexa Fluor 488 Invitrogen A11055). Images were captured using a Nikon eclipse E600 microscope with a Nikon digital camera DXM1200C using NIS elements BR2.30 SP4 imaging software (all from Nikon UK, Kingston-upon-Thames, United Kingdom).

Supplemental data

Supplemental Figure 1. Specificity of the antibody against human α-globin chains. Anti-human α-globin antibody was used (Santa Cruz D-16 sc-31110). Note a four bands ladder that recapitulates globin tetramer formation is observed under denaturating conditions.

Supplemental Figure 2. Measurement of human α-globin protein expression in mouse and humanised (normal and ΔMCS-R2) erythroblasts by flow cytometry. On the left, histogram plots. On the right, plots from flow cytometry analysis obtained with a goat IgG anti-human α-chains (Santa Cruz D-16 sc-31110) and Alexa Fluor 488 (Invitrogen A11055) conjugated anti-goat IgG.

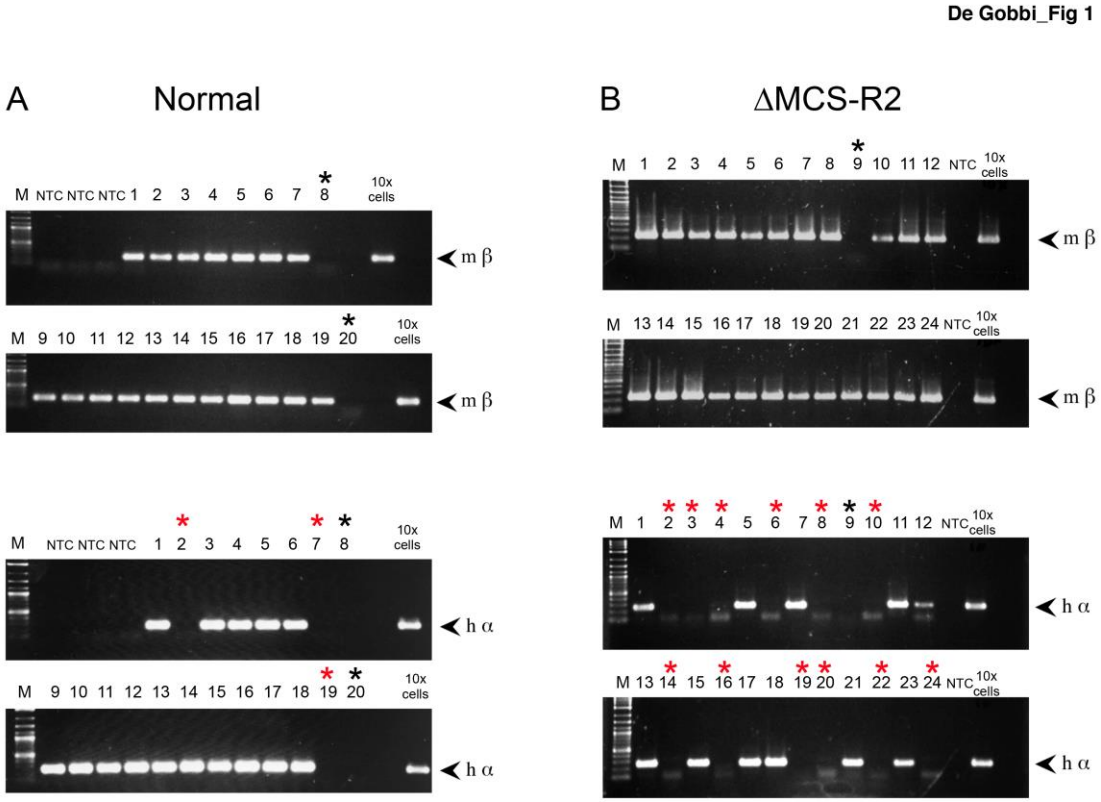

Fig. 1

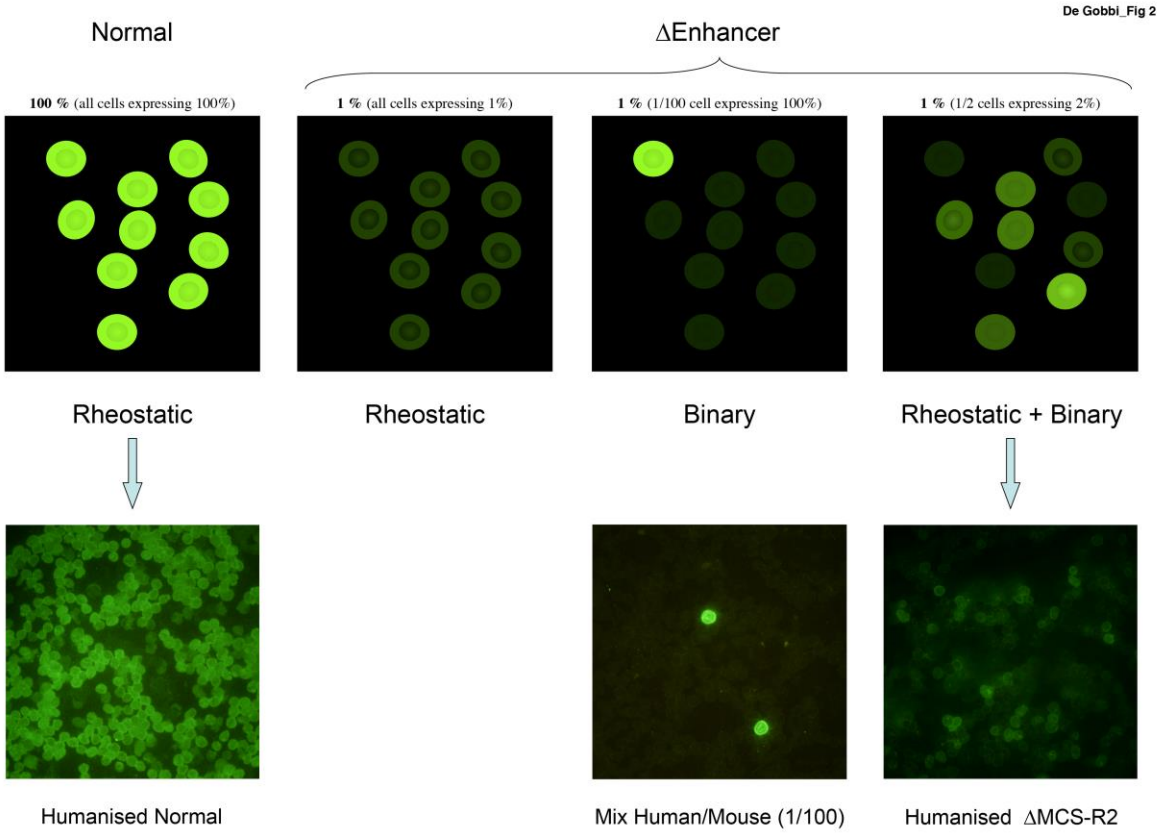

Fig. 2